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In breast cancer, the regulatory mechanisms that operate to control proper progression of the cell through each cell cycle are perturberd, leading to uncontrolled cell division. A key regulator of cell cycle passage is the G1 cyclin, cyclin E, which regulates the transition from G1 phase into S phase where replication of the DNA occurs. Cyclin E is synthesized and associates periodically with its catalytic subunit, the cyclin-dependent kinase CDK2, followed by its rapid destruction in early S phase. Abnormal accumulation of cyclin E is frequently observed in breast cancer. The levels of cyclin E correlate with the advanced stage and grade of the tumor and with a poor prognosis for breast cancer patients. Previous work has suggested that defects in the proteolytic destruction of cyclin E may account for its accumulation in these tumors. In the first year of this study, we have shown that the turnover of cyclin E is controlled by the ubiquitin-dependent SCF pathway. We have also isolated a novel human F-box protein, designated hCdc4, that specifically directs ubiquitination of cyclin E in a phosphorylation-dependent manner. In the second year of this proposal, we have extended the characterization of this newly identified pathway and we have addressed the question whether mutations to components of the pathway might account for accumulation of cyclin E in tumor cells.

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INTRODUCTION

A breast cell must pass through an ordered series of sequential phases, termed the cell cycle, before it divides into two daughter cells. The development of breast cancer is associated with multiple genetic alterations that disrupt the regulatory mechanisms of the cell cycle and ultimately lead to uncontrolled proliferation. Key regulators of the cell cycle are proteins, called cyclins, that are synthesized and destroyed periodically. Numerous studies have demonstrated that timed degradation of cyclin E is essential for proper cell cycle progression.

Abnormally high levels of cyclin E are frequently observed in breast cancer and have been shown to correlate with a poor prognosis for breast cancer patients (Gong *et al.*, 1994; Keyomarsi *et al.*, 1994; Keyomarsi *et al.*, 1995). It was shown that overexpression of cyclin E in transgenic mouse models can lead to mammary carcinoma (Bortner and Rosenberg, 1997). Furthermore, constitutive overexpression of cyclin E in cultured fibroblasts leads to genetic instability (Spruck et al., 1999). Thus, the inability of cells to degrade cyclin E could cause abnormal accumulation of cyclin E and may contribute to malignancy.

In the first year of this study we have identified the cellular factors that are involved in the turnover of cyclin E in human cells. We have shown that the ubiquitin-dependent degradation of cyclin E is governed by a recently identified ubiquitin E3 ligase, called SCF (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). We have also isolated a novel member of the F-box family of proteins, designated hCdc4, which acts as a specific receptor for cyclin E in its phosphorylated form. In the second and third year of this proposal, we have furthered the characterization of this pathway in human cells and we have investigated the hypothesis that accumulation of cyclin E previously observed in breast cancer-derived cell lines can be accounted for by mutations in components of the pathway.

BODY

Task 1: Defining the pathway that targets human cyclin E for degradation in yeast.
Task 2: Develop an *in vitro*-ubiquitination assay for cyclin E
Task 3: Define the pathway that targets cyclin E for degradation in mammalians

It was shown that human cyclin E is phosphorylated on threonine 380 and targeted to the proteasome by ubiquitination in the budding yeast *Saccharomyces cerevisiae* (Clurman *et al.*, 1996; Won and Reed, 1996). Phosphorylation of yeast G1 regulators, such as G1 cyclins, renders them susceptible to ubiquitin-dependent degradation by the recently identified SCF E3 ligase (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Cyclin E was found to be stabilizied in strains harboring temperature sensitive mutations in *cdc34*, *cdc53*, *skp1*, and *cdc4* . No stabilization of cyclin E was observed in a strain carrying a mutation in the *met30*, gene which encodes another F-box protein. These data strongly suggest that cyclin E is targeted by the Cdc34-dependent SCF pathway in yeast, and that Cdc4p is the relevant F-box protein acting as a specific adaptor for cyclin E.

Through BLAST searches, we identified a human homolog of the yeast Cdc4, termed hCdc4. We were able to demonstrate that phosphorylated cyclin E is targeted for ubiquitination and subsequent proteasomal degradation by the SCF^{hCdc4} ubiquitin ligase. Interestingly, in addition to

the previously identified phosphorylation site, threonine 380 (Clurman *et al.*, 1996; Won and Reed, 1996), we have discovered that a secondary site in cyclin E, threonine 62, is used to signal ubiquitination of cyclin E. We show that hCdc4 is rate-limiting for cyclin E turnover and most likely represents the predominant pathway that controls cyclin E levels in mammalian cells. The hCdc4 protein exists in at least 3 isoforms due to alternative splicing of the first amino-terminal exons encoding proteins of 110 kD (hCdc4α), and 70 kD (hCdc4β and hCdc4γ), respectively. The hCdc4α is ubiquituously expressed in all tissues examined whereas the expression of both hCdc4β and hCdc4γ seems to be tissue specific with high levels detected in sceletal muscle and brain, and to a lesser degree, heart.

Task 4, Determine if misregulation of cyclin E turnover accounts for cyclin E hyperaccumulation in breast cancer-derived cell lines

A panel of randomly chosen breast cancer-derived cell lines were anlayzed for expression of cyclin E protein. One cell line that showed extremely high cyclin E levels but had no amplification of the cyclin E gene (as determined by Northern blot) and had no mutations in the cyclin E gene (as determined by SSCP anlaysis), showed an aberrant hCdc4 transcript with lower mobility on a Northern blot. Careful mutational analysis of the hCDC4 genomic locus revealed a duplication of exons 8 and 9 leading to the introduction of a premature stop codon in the hCdc4 coding region. This results in the formation of a truncated hCdc4 protein that is non-functional since it can no longer bind to phosphorylated cyclin E. Concomitantly, cyclin E was found to be greatly stabilized in this cell line indicating an impairment of its degradation and thus, accounting for its accumulation. Furthermore, no ubiquitination activity using cyclin E as a substrate could be detected in an *in vitro* –ubiquitination assay with extracts prepared from the mutant cell line. The fact that no wild-type allele of the hCDC4 gene was detected suggests loss of heterozygosity (LOH) and thus, may qualify hCdc4 as a putative tumor suppressor. In an independent study, another group reported mutations in cell lines derived from breast and ovarian cancers (Moberg *et al.*, 2001).

Task 5, Identify mutations in the degradation pathway.

In order to find evidence for the involvement of a particular gene or protein in the pathogenesis of cancer, it is ultimately necessary to expand the analysis from cancer-derived cell lines to primary tumor specimens. To this end, we obtained samples of frozen endometrial adenocarcinomas and performed a mutational analysis of the hCDC4 gene. We chose to investigate endometrial cancers because it has been reported that cyclin E is frequently overexpressed in these tumors (Milde-Langosch *et al.*, 2001) and sufficient tissue was available to conduct a comprehensive study.

In brief, we found that 8 of a total of 51 endometrial adenocarcinomas analyzed contained hCDC4 gene mutations. These mutations were significantly correlated with high grade tumors and trended towards high stage tumors. Furthermore, most of the tumors did not retain a wild-type allele of hCDC4, indicative of loss of heterozygosity (LOH). The hCDC4 gene localizes to chromosome region 4q32 which is deleted at varying frequencies in many human cancers,

including breast cancer (Knuutila *et al.*, 1999). Strikingly, the frequency (16%) of hCDC4 gene mutations observed in our study matches closely the frequency (17%) of 4q32 deletions reported for endometrial adenocarcinomas (Knuutila *et al.*, 1999). These data suggest that hCdc4 may play a role as a tumor suppressor in the genesis of a broad range of human cancers. To test this hypothesis, we have now obtained breast tumor specimens and a mutational analysis is currently underway.

Unexpectedly, only 3 of 8 endometrial tumors with hCDC4 gene mutations showed elevated cyclin E protein levels when analyzed by Western blot, whereas 6 of 7 tumors accumulated the phosphorylated form of cyclin E. However, only 2 tumors exhibited both features, namely elevated cyclin E levels and accumulation of phosphorylated cyclin E, a scenario that would be expected from an impairment of cyclin E degradation processes.

Task 6, Final analysis

To further investigate the role of the different hCdc4 isoforms in the turnover of cyclin E, we designed siRNA oligo's, either specific for one the three isoforms, or targetting a common region. Preliminary data suggest that reducing cellular levels of hCdc4α and hCdc4γ interferes with cyclin E degradation. Interestingly, eliminating hCdcβ did not have an effect on overall cyclin levels. Expression of a siRNA oligo against exon3, shared by all three isoforms led to a similar accumulation of cyclin E as seen with hCdc4α and hCdc4γ siRNA. These results suggest that both hCdc4α and hCdc4γ are required for turnover of cyclin E.

Epitope tagged versions of the tree isoforms were ectopically expressed in 293 cells, and immunofluorescence was performed to determine their subcellular localization. hCdc4α and hCdc4γ were mainly localized in the nucleus, whereas hCdc4β was mainly found in the cytoplasm. This is consistent with their roles in cyclin E degradation, since cyclin E localization is limited to the nucleus.

KEY RESEARCH ACCOMPLISHMENTS

- Establishing the existence of a ubiquitin-dependent SCF pathway in mammalian cells.
- Identifying cyclin E as a target of this pathway.
- Cloning and functional characterization of hCdc4, a novel member of the F-box family of proteins.
- Developing an *in vitro* ubiquitination assay for cyclin E.
- Identification of a breast cancer-derived cell line that shows elevated cyclin E levels due to a defect in cyclin E proteolysis.
- A comprehensive analysis including Northern blot, Western blot, pulse-chase, SSCP, and *in vitro* ubiquitination assays clearly shows that inactivation of the hCDC4 gene leads to stabilization and subsequent accumulation of cyclin E.
- Loss of heterozygosity at the hCDC4 genomic locus suggests a role for hCdc4 as a tumor suppressor.
- Mutational analysis of endometrial adenocarcinomas yields a frequency of 16% of hCDC4 gene mutations, most of which exhibit loss of heterozygosity and thus, providing further indication for a role of hCdc4 as a tumor suppressor in a variety of human cancers.

- The hCdc4α and hCdc4γ isoforms, but not hCdc4β, are involved in degradation of cyclin E. Consistently, hCdc4α and hCdc4γ localize in the nucleus, where cyclin E is located.

REPORTABLE OUTCOME

A paper describing the identification of SCF^{hCdc4} as the predominant pathway that controls cyclin E turnover as well as the identification of an inactivating mutation in the hCDC4 gene in a breast cancer-derived cell line was published 2001 (Strohmaier *et al.*, 2001; and see Appendix).

A manuscript describing the results of a mutational analysis of endometrial adenocarcinomas has been published last year (Spruck *et al.*, 2002; and see Appendix).

CONCLUSIONS

The data obtained in in the timeframe of this award provide strong evidence for a predominant role of the SCF^{hCdc4} ubiquitin ligase in the ubiquitin-dependent degradation of human cyclin E. Mutations in the hCDC4 gene were detected in a breast cancer-derived cell line that showed elevated levels of cyclin E protein and in 16% of primary endometrial adenocarcinomas with either elevated cyclin E or accumulation of phosphorylated cyclin E or both. The fact that these mutations were accompanied by a coordinate loss of heterozygosity of the remaining wild-type allele suggests that hCdc4 may functions as a putative tumor suppressor in the genesis of many human cancers, including breast cancer. It is currently unclear how alterations of the hCDC4 gene that do not result in the accumulation of cyclin E contribute to the tumorigenic process. There is preliminary evidence which suggests that different isoforms of hCdc4 may target other cellular proteins, in addition to cyclin E, for degradation. Alternatively, hCdc4 may even serve other unknown functions. Disruption of these processes due to mutations in the hCDC4 gene may also play a role in tumorigenesis.

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APPENDICES

normal development. The mutations that disrupt its function *in vivo* reduce binding to Cyclin E and delay a decay in levels of Cyclin E. It has previously been shown that Cyclin E can be degraded in mammalian cells by direct interaction with a cullin[17–19]. Our findings, together with the observation that mutations in the *C. elegans* genes *cul1* and *lin-23* (which encode a cullin and an F-box protein respectively) have increased cell divisions[20,21], highlight the importance of SCF-mediated degradation in regulating cell proliferation through Cyclin E. Because *ago* RNA is expressed in a dynamic pattern, our results indicate that degradation of Cyclin E is not constitutive *in vivo*. Dynamic expression of Ago provides another mechanism by which cyclin/cdk activity and cell proliferation can be regulated during development. Finally, we implicate impaired proteolysis of Cyclin E in the pathogenesis of human cancers. □

Methods

Fly stocks

All crosses were conducted at 25 °C. *w; FRT80B* males were mutagenized with ethylmethanesulphonate (EMS), then crossed to *y w eyFLP;FRT80B P[mini-w, arm-LacZ]* virgin females (stocks a gift of J. Treisman). Males with more white than red eye tissue were selected and maintained as balanced stocks. Alleles of *archipelago* isolated were ago^1, ago^3 and ago^4. Other stocks were *y w hsFLP;FRT80B P[πMyc] P[w y], w;FRT80B P[mini-w] P[UbiGFP]/TM6B* (a gift of B. Edgar), *GMR-p35* (a gift of K. White) and *w;* $cycE^{JP}$ (a gift of H. Richardson).

Microscopy, immunohistochemistry, flow cytometry

Adult eyes were photographed submerged in mineral oil. Imaginal disc tissue of the indicated genotypes was fixed and stained for Cyclin E and β-galactosidase as described previously[3]. Images were collected on a Carl Zeiss Axiovert 100M Confocal microscope. The mouse monoclonal antibody to *Drosophila* type I Cyclin E and the Cyclin B antibody were gifts of H. Richardson and C. Lehner, respectively. The antisense *Drosophila cyclin E* probe was derived from full-length type I *cyclin E* complementary DNA. The antisense *ago* probe was derived from a full-length cDNA. Flow cytometry on third-instar larval wing discs was performed as described previously[3]. For *ago* loss-of-function FACS analysis, the following genotype was used: *y w hsFLP; FRT80B* ago^3*/FRT80B P[mini-w] P[UbiGFP]*.

Molecular biology

For GST-fusion proteins, PCR fragments corresponding to the C-terminal 660 amino acids of the wild-type or *ago* mutant open reading frames were cloned in-frame into the pGEX-2T vector (Amersham Pharmacia). Following induction, equal amounts of intact GST–AgoΔN fusion proteins were incubated with 100 μg of S2 whole-cell extract from cells transfected using the CELLFECTIN reagent (Gibco BRL) with *Drosophila cdk2* and Myc-epitope-tagged *Drosophila* type I *cyclin E* cDNAs cloned into pIE1–4 insect expression vectors (Novagen). Myc-tagged cyclin E protein was detected in western blots using the 9E10 anti-Myc tag monoclonal antibody; human Cyclin E was detected in lysates of human ovarian cancer cell lines synchronized in G1/S by incubation for 36 h in 2 mM thymidine with the HE12 anti-cyclin E monoclonal antibody (both antibodies were a gift of E. Harlow). For northern analysis, 10 μg of total cellular RNA was probed with a 0.9-kilobase *Bam*H1-*Xmn*1 fragment of the human *cyclin E* cDNA.

Characterization of human *ago*

The α and β forms of human *ago* were identified in BAC067826 by exon-prediction programmes. The existence of both forms was confirmed by RT-PCR. An in-frame termination codon is present 75 nucleotides upstream from the initiating ATG in the β cDNA. We were unable to locate an upstream in-frame termination codon in the α cDNA. These sequences have been deposited in GenBank. Human *ago* was amplified by RT-PCR in six overlapping fragments. PCR products were resolved by gel electrophoresis and sequenced directly with the BigDyeTerminator kit (Applied Biosystems) and analysed on an ABI300 genetic analyser. In addition to eight primary tumours, the cancer cell lines analysed were: breast (MCF7ADR, MDAMB435, T47D, BT483, MDAMB436, MDAMB453, MDAMB468, MDAMB415, MDAMB231, MDAMB175, MDAMB157, HS157, HS467T, HS496T, HS578T, UACC893, BT549), ovarian (ES-2, IGROV-1, MDAH2774, OV1063, OVCAR3, OVCAR4, OVCAR5, OVCAR8, SKOV3, SW626), lung (NCIH460, NCI522, HOP92), central nervous system (SF295, SNB19, U251), leukaemia (CCRF-CEM, K562, MOLT4, RPMI-8226, SR), colon (COLO205, HCT116, HCT15), renal (786-0, ACHN, CAKI-1, SN12C, U031), melanoma (LOXMVII, M14, SKMEL2, UACC62) and osteosarcoma (U2OS, SAOS2). The wild-type controls were EBV-immortalized cell lines from normal individuals[22].

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Correspondence and requests for materials should be addressed to I.K.H. (e-mail: hariharan@helix.mgh.harvard.edu). The sequences for human Ago have been deposited in GenBank under accession numbers AF411971 (α form) and AF411972 (β form).

Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line

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Cyclin E, one of the activators of the cyclin-dependent kinase Cdk2, is expressed near the G_1–S phase transition and is thought to be critical for the initiation of DNA replication and other S-phase functions[1–3]. Accumulation of cyclin E at the G_1–S boundary is achieved by periodic transcription coupled with regulated proteolysis linked to autophosphorylation of cyclin E[4]. The proper timing and amplitude of cyclin E expression seem to

be important, because elevated levels of cyclin E have been associated with a variety of malignancies[5,6] and constitutive expression of cyclin E leads to genomic instability[7]. Here we show that turnover of phosphorylated cyclin E depends on an SCF-type protein-ubiquitin ligase that contains the human homologue of yeast Cdc4, which is an F-box protein containing repeated sequences of WD40 (a unit containing about 40 residues with tryptophan (W) and aspartic acid (D) at defined positions). The gene encoding hCdc4 was found to be mutated in a cell line derived from breast cancer that expressed extremely high levels of cyclin E.

We and others have previously demonstrated that human cyclin E is targeted for ubiquitin-mediated proteolysis in both mammalian and yeast cells by phosphorylation of residue Thr 380 (refs 8, 9). Because the characteristics of cyclin E turnover were found to be similar in mammalian and yeast cells, we used yeast to elucidate the cellular machinery that targets cyclin E for proteolysis in human cells. In yeast, a protein-ubiquitin ligase system known as SCF has been shown to target a number of proteins for ubiquitin-mediated proteolysis in a phosphorylation-dependent manner[10–12]. SCF consists of four subunits: Skp1, Cdc53/Cul-1, Roc1 and one of a family of F-box proteins, which determine substrate specificity[13]. The suggestion that SCF might be involved in turnover of cyclin E is consistent with the observation that levels of cyclin E are elevated in *Cul1*-deficient embryos[14,15]. To determine whether SCF is indeed involved in cyclin E turnover in yeast, *cdc53* and *skp1* thermosensitive mutants were compared with a wild-type strain for cyclin E turnover at the restrictive temperature (Fig. 1a). Cyclin E was stabilized in both the *cdc53* and *skp1* thermosensitive mutants, indicating that SCF activity is required for cyclin E turnover in yeast. The ubiquitin-conjugating (E2) enzyme that usually works in concert with SCF in yeast is Cdc34. Accordingly, *cdc34* mutants were also found to stabilize cyclin E (Fig. 1a). In yeast, the three best-characterized F-box proteins are Cdc4, Grr1 and Met30. Whereas cyclin E turned over at the wild-type rate in a *met30* mutant, it was stabilized in a *cdc4* mutant (Fig. 1a). Thus, in yeast, ubiquitination of cyclin E is most probably mediated by the concerted action of the ubiquitin-conjugating enzyme Cdc34 and the protein-ubiquitin ligase SCF^{Cdc4}. One caveat with this interpretation is that stabilization of the CDK-inhibitor Sic1 in a *cdc4* mutant might prevent phosphorylation of cyclin E, thereby conferring stabilization indirectly. We found, however, that the turnover rate

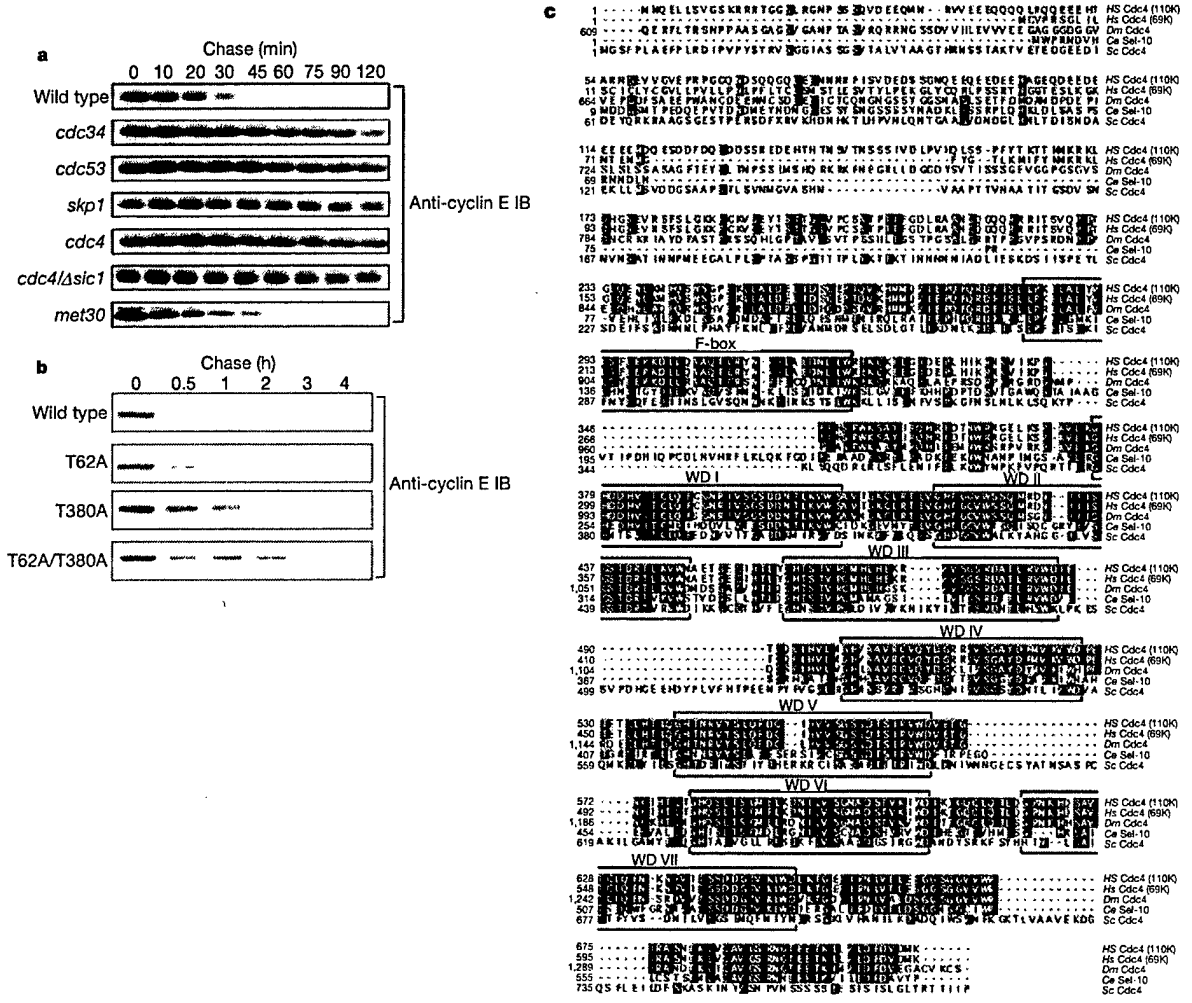

Figure 1 Human cyclin E is targeted for ubiquitin-dependent degradation by the SCF^{Cdc4} protein-ubiquitin ligase in yeast. **a**, Cyclin E was expressed from the inducible *GAL1* promoter in a wild-type strain and in various thermosensitive SCF mutant strains at the restrictive temperature, and the turnover of cyclin E was followed by immunoblotting (IB). **b**, Wild-type cyclin E and phosphorylation-site mutants of cyclin E were expressed from the *GAL1* promoter in yeast. Analysis was as in **a**. **c**, Protein sequence alignment showing (from top to bottom) the two hCdc4 isoforms (*Hs* 110K and 69K), a homologue from *Drosophila* (*Dm* Cdc4), a homologue from *C. elegans* (*Ce* Sel-10), and yeast Cdc4 (*Sc* Cdc4). Identical amino acids are highlighted in black, and conserved substitutions are highlighted in grey. The F-box and the seven WD40 repeats are boxed.

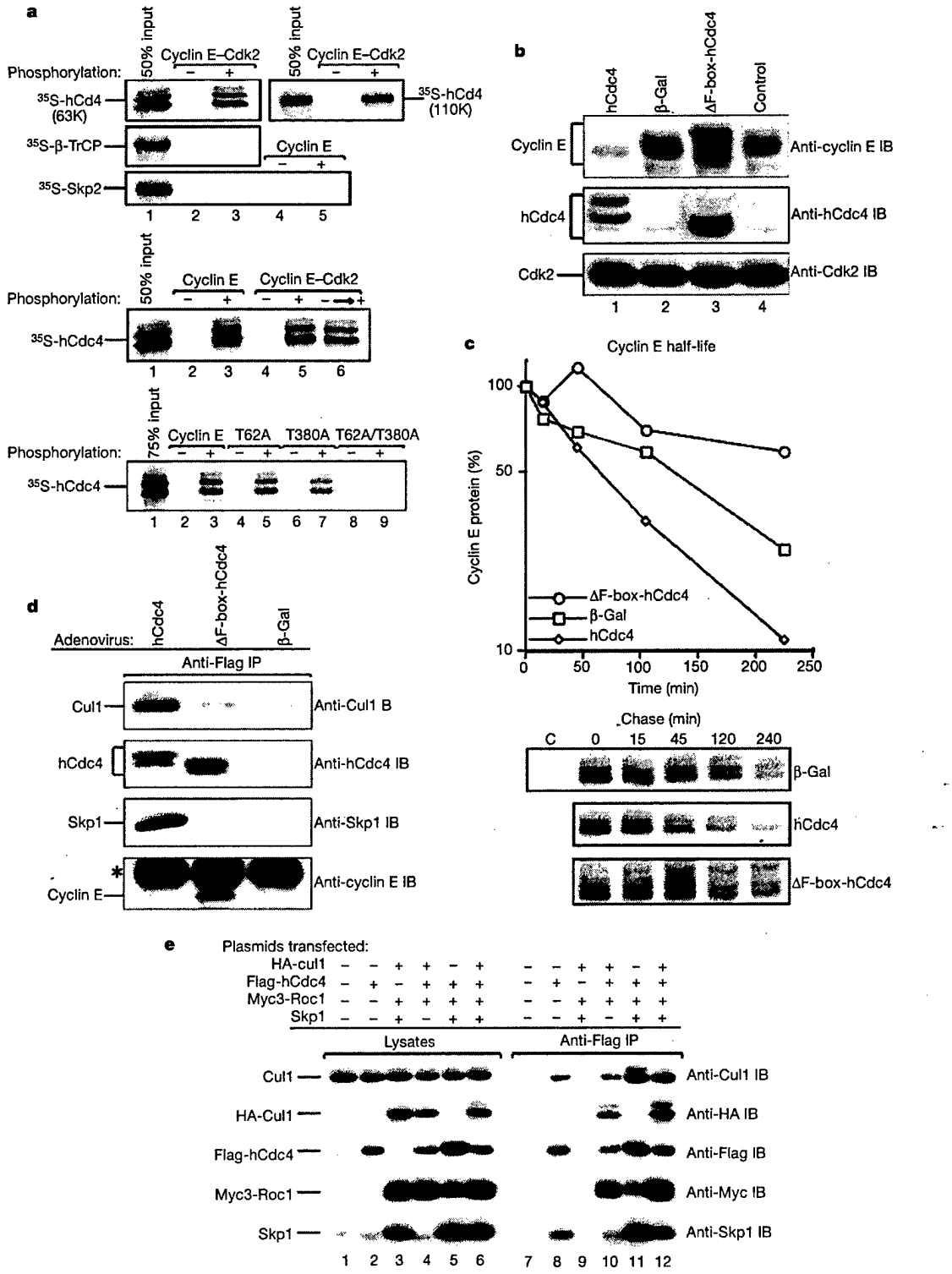

Figure 2 Human Cdc4 assembles into SCF complexes with Cul1, Skp1 and Roc1 *in vivo*, and regulates cyclin E turnover through specific association with phosphorylated cyclin E. **a**, *In vitro* translated, ^{35}S-labelled hCdc4 (63K and 110K forms), β-TrCP and Skp2 were assayed for binding to either free or Cdk2-bound GST-tagged cyclin E purified from SF9 insect cells on glutathione beads (lanes 1–5, top and middle panels). Dephosphorylation of cyclin E was performed after purification with λ phosphatase. ^{35}S-labelled hCdc4 was also tested for binding to cyclin E that had been dephosphorylated followed by rephosphorylation by its associated kinase, Cdk2, in the presence of 1 mM ATP (lane 6, middle panel), and for binding to single phosphorylation-site mutants (T62A, T380A) as well as the double mutant (T62A/T380A) (bottom panel). **b**, Thymidine-arrested KB cells were transduced with recombinant adenoviruses expressing hCdc4 (lane 1), a ΔF-box hCdc4 (lane 3) or a control virus (β-galactosidase) (lane 2). Lane 4 is an uninfected control. **c**, ^{35}S-methionine pulse-chase analysis of cyclin E in adenovirally transduced KB cells described in **b**. **d**, Immunoprecipitation (IP) of adenovirally transduced wild-type and mutant hCdc4 from KB cells described in **b**, and analysis of coprecipitated proteins by western blotting. In the bottom panel, the asterisk corresponds to the immunoglobulin-γ (IgG) heavy chain. **e**, 293T cells were transfected with the indicated plasmids and lysates were used for anti-Flag immunoprecipitations. Immune complexes (lanes 7–12) or crude lysates (lanes 1–6) from each transfection were analysed for the presence of Cul1, Skp1, Roc1 and hCdc4 by immunoblotting (IB).

was unchanged in a *cdc4/sic1* double mutant relative to the *cdc4* mutant (Fig. 1a), confirming that Cdc4 is indeed the critical F-box protein for cyclin E degradation in yeast.

Close scrutiny of half-life data obtained for the T380A mutant and comparison with data for wild-type cyclin E in SCF mutants suggested that the T380A mutant may still be susceptible to SCF-mediated ubiquitination and proteolysis. Accordingly, mutations were constructed at other potential phosphorylation sites. We found that the T62A mutation rendered cyclin E slightly more stable than the wild type (Fig. 1b). Significantly, the double mutant (T62A/T380A) was more stable than the T380A mutant (Fig. 1b). This suggests that T62 is a secondary phosphorylation site involved in ubiquitination and turnover of cyclin E.

We found a human expressed-sequence tag (EST) in the EST database of GenBank (http://www.ncbi.nlm.nih.gov) that, when translated, had significant homology to yeast Cdc4 (Fig. 1c). Analysis of hCdc4 complementary DNAs from a number of cell lines and the genomic structure of the *hCDC4* locus indicated that

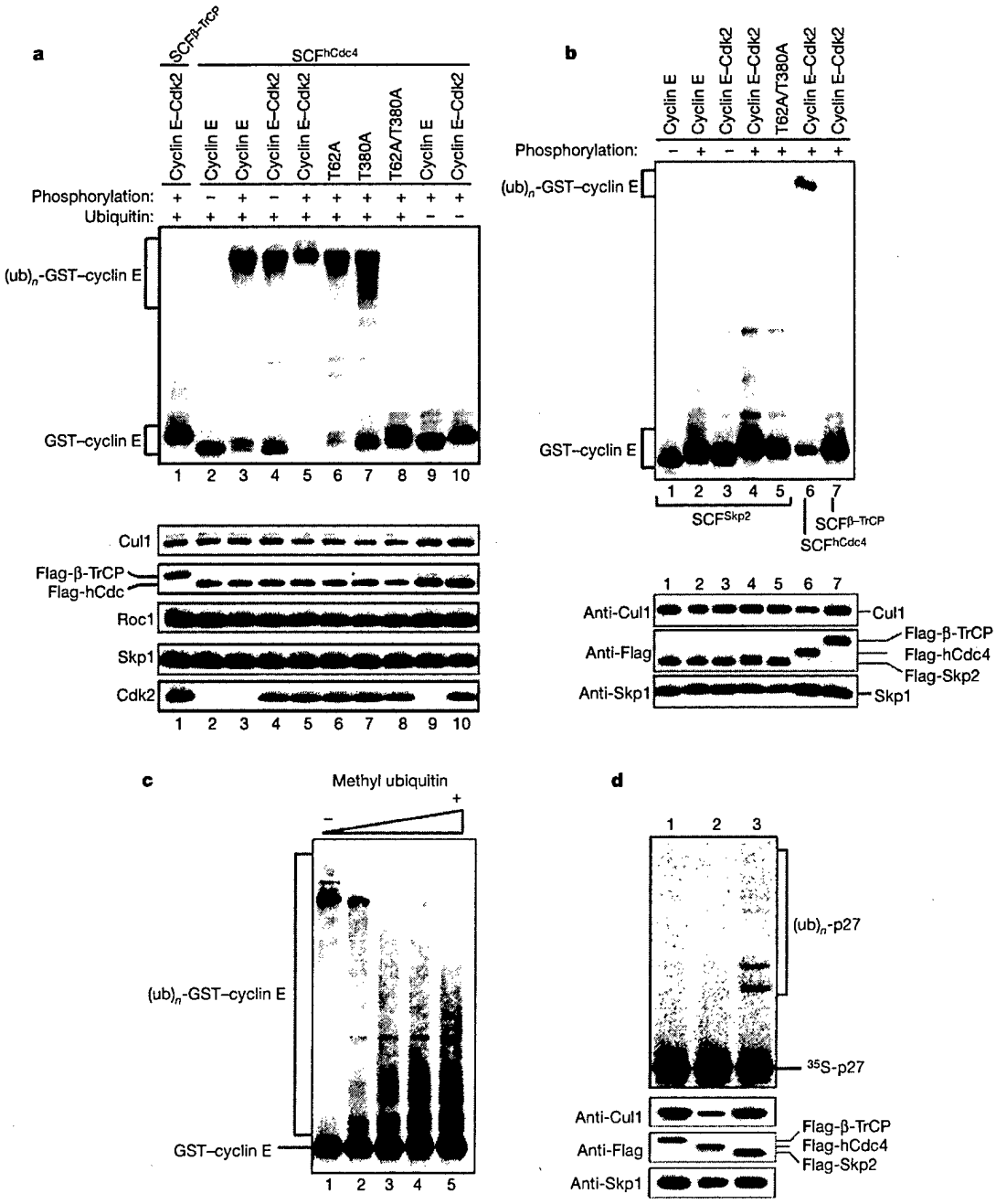

Figure 3 SCF^{hCdc4} ubiquitinates cyclin E in a phosphorylation-dependent manner *in vitro*. **a**, Anti-Flag immunoprecipitates from 293T cells transfected with either SCF^{hCdc4} (lanes 2–10) or $SCF^{\beta\text{-}TrCP}$ (lane 1) were assayed for the ability to ubiquitinate cyclin E and phosphorylation-site mutants (top panel). The bottom panel shows anti-Flag immunoprecipitates. **b**, Comparison of immunoprecipitated SCF^{Skp2} (lanes 1–5), SCF^{hCdc4} (lane 6) and $SCF^{\beta\text{-}TrCP}$ (lane 7) for the ability to ubiquitinate cyclin E (top panel). ub, ubiquitin. Immunoblots for Cul1, Skp1 and Flag-tagged F-box proteins are shown (bottom panel). **c**, Effect of increasing concentrations of methylated ubiquitin on the mobility of poly-ubiquitinated cyclin E species. **d**, Comparison of the ability of immunoprecipitated SCF complexes to ubiquitinate $p27^{Kip1}$.

two alternatively spliced variants exist that encode proteins with different amino termini (Fig. 1c, top two lines). A search for related proteins in other species suggested that homologues exist in *Drosophila melanogaster* as well as *Caenorhabditis elegans* (Fig. 1c). *In vitro* translation of the *hCDC4* cDNA produced a polypeptide with a relative molecular mass of about 63,000 (M_r 63K). However, further analysis of *hCDC4* transcripts and genomic structure indicated that two alternative forms of the protein exist. The 63K polypeptide derived from the initial hCdc4 EST is a slightly truncated version of a 69K species, which appears to be quite tissue specific (see below). The prevalent species in most tissues, consisting of 707 amino acids, runs aberrantly on SDS gels at an apparent M_r of 110K (see below). To generate the 110K form, one large upstream coding exon is substituted for the first exon of the 69K species (data not shown). We found that expression of the 110K form of hCdc4 could partially rescue a *cdc4* mutation in the yeast *Saccharomyces cerevisiae* (data not shown), consistent with these proteins being functional and structural homologues.

To determine whether hCdc4 interacts specifically with phosphorylated cyclin E, hCdc4 translated *in vitro* (the 63K and 110K forms) was incubated with glutathione beads bound to either free glutathione *S*-transferase (GST)–cyclin E complexes or GST–cyclin E–Cdk2 complexes. In parallel samples, cyclin E was either phosphorylated or dephosphorylated. hCdc4 binds to phosphorylated free or Cdk2-bound cyclin E, but not to dephosphorylated cyclin E, regardless of Cdk2 binding (Fig. 2a). In contrast, *in vitro* translated β-TrCP, another human WD40-repeat-containing F-box protein, bound to neither phosphorylated nor dephosphorylated cyclin E (Fig. 2a). It has been suggested that the human F-box protein Skp2, which contains leucine-rich repeats rather than WD40 repeats, targets cyclin E for ubiquitin-dependent degradation[16,17]. However, *in vitro* translated Skp2 bound to neither phosphorylated nor dephosphorylated cyclin E, either free or bound to Cdk2 (Fig. 2a). hCdc4 (63K) translated *in vitro* was assayed further for the ability to bind to cyclin E phosphorylation site mutant proteins. Cyclin E (T380A) was subjected to phosphorylation and dephosphorylation and assayed for hCdc4 binding as had been done for wild-type cyclin E. Binding of this mutant protein was reduced but not eliminated (Fig. 2a). However, on the basis of our analysis presented in Fig. 1b, we assayed both the T62A (single) and T62A/T380A (double) mutants for hCdc4 binding. Cyclin E (T62A) was only slightly reduced in hCdc4 binding (Fig. 2a). However, cyclin E (T62A/T380A) was completely defective in binding (Fig. 2a), consistent with the *in vivo* half-life data (Fig. 1b).

To determine whether expression of hCdc4 *in vivo* has an impact on cyclin E turnover, KB cells were transduced with an hCdc4 recombinant adenovirus. Endogenous levels of cyclin E were

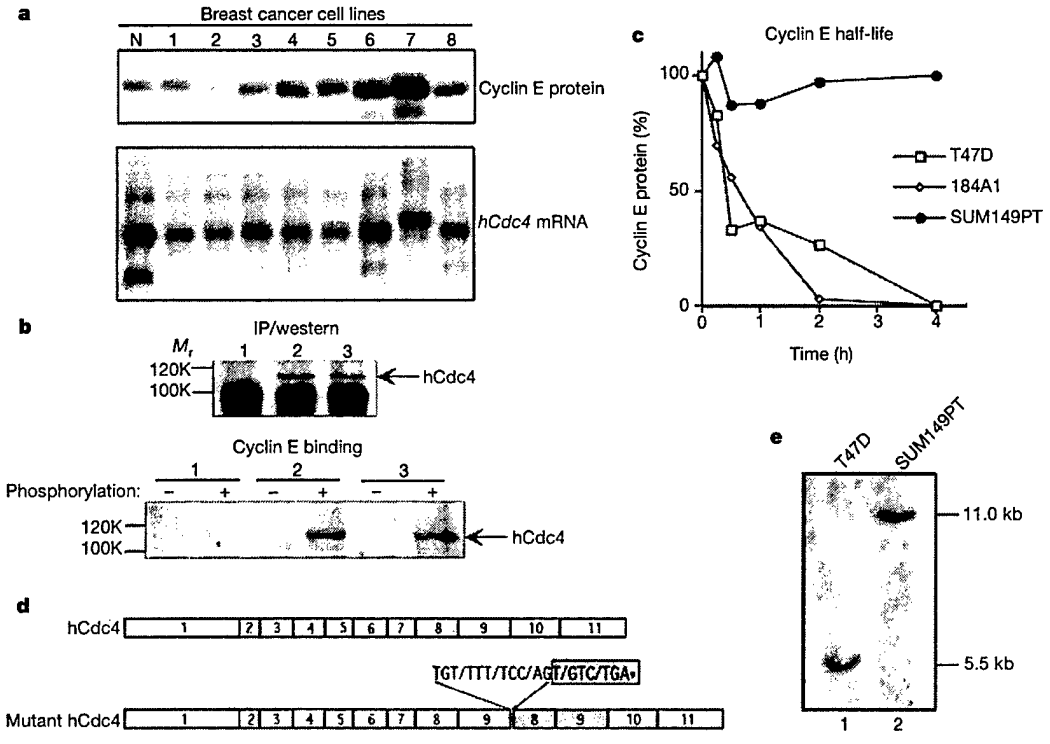

Figure 4 Aberrant hCdc4 mRNA, loss of hCdc4 protein and loss of heterozygosity in cell lines derived from breast cancer with high cyclin E expression. **a**, Eight randomly chosen breast-cancer-derived cell lines (lanes 1–8) and a breast epithelial cell line (184A1, lane N) were analysed for cyclin E expression by immunoblotting (top panel), and for hCdc4 transcripts by northern blot analysis (bottom panel). Cell lines: lane 1, MDA-MB-435S; lane 2, T47D; lane 3, BT-549; lane 4, ZR-75-1; lane 5, MDA-MB-436; lane 6, MDA-MB-157; lane 7, SUM149PT; lane 8, SK-BR-3. **b**, The breast epithelial cell line 184A1 (lane 2) and the breast-cancer-derived cell lines SUM149PT (lane 1) and T47D (lane 3) were analysed for expression of hCdc4 protein by anti-hCdc4 immunoprecipitation (IP) followed by immunoblotting using specific anti-hCdc4 antibodies (top panel) or by incubating crude lysates prepared from these cell lines with either dephosphorylated (control) or phosphorylated GST–cyclin E immobilized on glutathione beads followed by SDS–PAGE and western blotting with anti-hCdc4 antibodies (bottom panel). The heavy band migrating ahead of hCdc4 in the immunoprecipitation–immunoblot experiment corresponds to IgG heavy chain–light chain heterodimers. **c**, ^{35}S-methionine pulse-chase analysis was performed to measure the turnover rate of cyclin E in the indicated cell lines. **d**, Structure of SUM149PT hCdc4 cDNA. Exons are numbered. Shaded exons are duplicated in tandem resulting from a tandem genomic duplication of the region containing exons 8 and 9. Spliced intronic sequences in the cDNA are shown, which lead to a chain termination at the beginning of the duplicated exon 8. **e**, Southern blot analysis of genomic DNA from breast-cancer-derived cell lines T47D and SUM149PT.

dramatically reduced compared with control adenovirus transductions (Fig. 2b). Conversely, when an adenovirus expressing an F-box-deleted (ΔF-box) and thereby, most probably, dominant negative hCdc4 allele, was transduced into the same cell line, a significant accumulation of cyclin E was observed (Fig. 2b). In addition, the bulk of accumulated cyclin E was hyperphosphorylated on the basis of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) mobility (Fig. 2b), consistent with a block in the degradation specifically of phosphorylated cyclin E. ^{35}S-methionine pulse-chase experiments were performed on parallel adenoviral transductions (Fig. 2c). Transduction of wild-type hCdc4 led to a decrease in cyclin E half-life, whereas transduction of the dominant negative hCdc4 allele led to an increase in cyclin E half-life (Fig. 2c). Thus hCdc4 levels are rate limiting for cyclin E turnover. To confirm that the ΔF-box version of hCdc4 indeed had the appropriate characteristics to behave as a dominant negative, we showed by immunoprecipitation that it binds specifically to phosphorylated cyclin E *in vivo* but not to components of SCF (Fig. 2d).

To confirm that wild-type hCdc4 is part of an SCF complex, Flag-tagged hCdc4 was introduced into 293T cells by transfection. Analysis of anti-Flag immunoprecipitates indicated that hCdc4 is associated with both endogenous and co-transfected core components of human SCF (Fig. 2e).

To determine whether SCF^{hCdc4} can ubiquitinate cyclin E in a phosphorylation-dependent manner, expression plasmids for Flag-hCdc4, as well as the other three components of SCF, were cotransfected into 293T cells. Anti-Flag immunoprecipitates were then tested for their ability to ubiquitinate phosphorylated cyclin E. Immunoprecipitated SCF^{hCdc4} was capable of efficiently ubiquitinating phosphorylated cyclin E, either free or bound to Cdk2 (Fig. 3a, b). Addition of methylated ubiquitin increased the mobility of the cyclin E derivatives (Fig. 3c), confirming that the modification is indeed ubiquitination. In parallel experiments using Flag-tagged β-TrCP and Skp2, respectively, anti-Flag immunoprecipitates were incapable of efficiently ubiquitinating either phosphorylated or dephosphorylated cyclin E even though SCF complexes were formed (Fig. 3a, b). In contrast, immunoprecipitated SCF^{Skp2} could ubiquitinate phosphorylated $p27^{Kip1}$, one of its established targets (Fig. 3d). Consistent with the binding studies described above, SCF^{hCdc4}-mediated ubiquitination of cyclin E (T62A) was slightly reduced compared with wild-type cyclin E, ubiquitination of cyclin E (T380A) was moderately reduced, and the double mutant was not ubiquitinated at all (Fig. 3a). Thus hCdc4 is incorporated into an SCF complex that efficiently ubiquitinates phosphorylated but not unphosphorylated cyclin E. These data, taken together with the ability of transduced wild-type and dominant negative hCdc4 to affect dramatically the steady-state levels of cyclin E *in vivo*, strongly suggest that SCF^{hCdc4} represents the predominant pathway mediating turnover of cyclin E in mammalian cells.

A tissue RNA blot indicated that hCdc4 is expressed in most, if not all, tissues. In most tissues, the predominant messenger RNA species is about 5.5 kilobases (kb) long (data not shown). However, some tissues strongly expressed a 4-kb mRNA as the predominant form, in particular brain and skeletal muscle. The fact that hCdc4 is expressed at high levels in non-proliferating tissues suggests a function in addition to turnover of cyclin E, because cyclin E expression should be limited to tissues undergoing cell division. These data also suggest that the 5.5-kb mRNA, which is ubiquitously expressed, encodes the F-box protein responsible for targeting cyclin E. Using exon-specific probes, we have shown that the 5.5-kb mRNA encodes the 110K hCdc4 isoform, whereas the 4-kb species encodes the 69K species (data not shown). Analysis of hCdc4 expression in synchronized HeLa cells indicated that neither hCdc4 mRNA nor protein is regulated during the cell cycle (data not shown).

Levels of cyclin E are elevated in many types of human malignancy[5,6]. Furthermore, dysregulation of cyclin E levels has been directly linked to genomic instability[7] and tumorigenesis in model systems[18]. To determine whether loss of hCdc4 might account for elevated levels of cyclin E, we first analysed a panel of cell lines derived from breast cancer for cyclin E levels (Fig. 4a). Two such cell lines (MDA-MB-157 and SUM149PT, lanes 6 and 7, respectively) exhibited significant elevation of cyclin E above the level observed in 184A1 (ref. 19), an immortalized, non-transformed breast epithelial cell line (Fig. 4a, lane N). One of the cell lines expressing high levels of cyclin E (MDA-MB-157) has been shown previously to contain a genomic amplification of the cyclin E locus[20]. Northern blot analysis using the hCdc4 cDNA as a probe (Fig. 4a) indicated that the 184A1 breast epithelial cell line contained a predominant hybridizing mRNA species at approximately 5.5 kb and a species with lower abundance at about 4 kb. Most of the breast cancer cell lines expressed only the 5.5-kb species. However, one cell line (SUM149PT) that exhibited high levels of cyclin E expressed an mRNA species of reduced mobility. The lack of any of the mRNA species characteristic of hCdc4 suggests a mutational lesion and, furthermore, loss of heterozygosity.

To determine whether the aberrant mRNA species in the SUM149PT cell line corresponded to a loss or alteration of hCdc4 protein, hCdc4 was concentrated from lysates either by adsorption to immobilized phosphorylated cyclin E or by immunoprecipitation with anti-hCdc4 antibody. The concentrated hCdc4 was then, in each case, subjected to SDS–PAGE and western blotting with anti-hCdc4 antibody (Fig. 4b). The 184A1 cell line and a breast cancer cell line that does not exhibit elevated cyclin E levels (T47D) contained the 110K hCdc4 isoform, which was detected either by binding specifically to phosphorylated cyclin E or by immunoprecipitation with anti-hCdc4 antibody. However, SUM149PT expressed no hCdc4-crossreactive protein capable of being immunoprecipitated or that bound to phosphorylated cyclin E within the limit of detection. ^{35}S-methionine pulse-chase experiments support this interpretation in that cyclin E has an extended half-life in the SUM149PT cell line compared with 184A1 and T47D cell lines (Fig. 4c).

To determine the nature of the mutation at the *hCDC4* locus in the SUM149PT cell line, the presumptive protein-coding region of the cDNA was sequenced and found to contain a direct repeat of exons 8 and 9 separated by 11 base pairs of intronic sequence (Fig. 4d). This mutation would be predicted to result in chain termination, eliminating the last four (of seven) WD40 repeats, presumably rendering the resulting polypeptide nonfunctional. Indeed, translation *in vitro* of the cDNA isolated from the SUM149PT cell line produced a truncated product that did not bind to phosphorylated cyclin E (data not shown). The loss of heterozygosity and internal genomic duplication at the *hCDC4* locus was confirmed by Southern blotting (Fig. 4e). DNA was cleaved with *Sst*I, which cuts in intronic sequences immediately downstream of exon 7, and with *Eco*RV, which cuts in intronic sequences immediately downstream of exon 10, and probed with a genomic fragment containing exons 8 and 9. The predicted wild-type fragment is 5.5 kb long, whereas that of the mutant is close to 11 kb. This finding and the implication of elevated cyclin E in carcinogenesis suggests that hCdc4 may be a tumour suppressor associated with some types of malignancy, including breast cancer. hCdc4 was identified independently on the basis of homology to the *Drosophila archipelago* gene product, also shown to regulate cyclin E proteolysis, and given the name human Ago in that study[21]. □

Methods

Plasmids and baculovirus constructions

A human EST encoding part of the hCdc4 gene was amplified from HeLa mRNA by polymerase chain reaction with reverse transcription (RT-PCR) using two sequence-

specific oligonucleotide primers, Pcr1 (5′-gcaagcttatgggtttctacggcacatt-3′, forward), and Pcr2 (5′-atgggccctgctcttcacttcatgtcc-3′, reverse), and TA cloned into pCR2.1 (Invitrogen). The sequence of the cloned cDNA was verified in its entire length (1.7 kb) by sequencing and found to match the sequence published in the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank, Genbank accession number BAA91986.1). For further details, see Supplementary Information. A mammalian transfection plasmid expressing N-terminal Flag-tagged hCdc4 protein was constructed by subcloning into pFLAG-CMV2 (Sigma). For expression in *Escherichia coli*, hCdc4 was tagged at the N terminus with a RGS.His epitope through subcloning into pQE-10 (Qiagen). Complementary DNAs encoding hCdc4 and a ΔF-box mutant that had been deleted for its F-box by a two-step PCR protocol, as well as the cDNA coding for β-galactosidase, were cloned into pDV46.

Recombinant adenoviruses were generated by co-transfecting the recombinant plasmids and pBHG10 (ref. 22) into 293 cells using the calcium phosphate precipitation method. The β-TrCP and Skp2 clones were gifts of F. Mercurio and M. Pagano, respectively, and were cloned into pFLAG-CMV2 to obtain β-TrCP and Skp2 tagged at their N termini with the Flag epitope. The mammalian transfection plasmid pCDNA3-Cul1-HA was a gift of R. Klausner, and pCDNA3-3MYCROC1 as well as pCDNA3-hSkp1 were gifts of Y. Xiong. Baculovirus expressing cyclin E with a GST tag at its N terminus was a gift of B. Sarcevic. Recombinant baculoviruses expressing GST-tagged versions of cyclin E phosphorylation site mutants (T62A, T380A, T62A/T380A) were generated using the pFastBac-system (Gibco BRL) according to the manufacturer's protocol. Baculovirus encoded proteins were expressed in SF9 insect cells grown in Ex-Cell 401 media (JRH) supplemented with 2% fetal bovine serum.

Analysis of cyclin E turnover in yeast

All yeast strains are isogenic to 15Daubᴧ, a *bar1Δura3Δns*, a derivative of BF264-15D (ref. 23). Several thermosensitive *skp1* mutants with different cell cycle arrest phenotypes were constructed by a combination of PCR mutagenesis and *in vivo* gap repair similar to the procedure described by Muhlrad *et al.*[24]. The mutant shown in Fig. 1 (*skp1-24*) arrested with 1C DNA content and a multi-budded phenotype. To analyse turnover of cyclin E in various yeast mutants expressing cyclin E from the inducible *GAL1* promoter, cells were grown in YEP-raffinose at 25 °C to an absorbance at wavelength 600 nm of 0.3. Cells were then shifted to 35 °C and after 30 min galactose was added to a final concentration of 2% to induce the *GAL1* promoter. To terminate cyclin E expression after 60 min, cells were collected on filters and transferred to YEPD media and incubation was continued at 35 °C. Extracts were prepared from aliquots taken after the periods indicated and analysed for cyclin E by western blot. For further details, see Supplementary Information.

Cell culture and immunological techniques

A panel of cell lines derived from breast cancer was obtained from the American Type Culture Collection (ATCC) and the University of Michigan Breast Cell/Tissue Bank and Database; the cells were grown in media recommended by the suppliers. HeLa, KB (human epidermoid carcinoma) and 293T cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified 37 °C incubator with 5% CO_2. 293T cells were transfected with various combinations of plasmids in 10-cm dishes by the calcium phosphate precipitation method. Forty hours after transfection, cells were lysed and subjected to immunoprecipitation followed by immunoblotting. For further details, see Supplementary Information.

Pulse-chase, northern and Southern blot analyses

Pulse-chase experiments were performed on thymidine-arrested cells as described[25]. Thymidine-arrested KB cells were co-transduced with adenovirus encoding cyclin E (resulting in a five- to tenfold elevation over endogenous levels) and virus encoding either wild-type hCdc4, a ΔF-box hCdc4, or control β-galactosidase. Viral transductions were incubated for 24 h before pulse-chase. Immunoprecipitations were performed with a monoclonal anti-cyclin E antibody (HE172). Quantification of bands was performed with ImageQuant software (Molecular Dynamics). For northern blot analysis, 2 μg of poly(A)$^+$ RNA was isolated from asynchronously growing cultures according to the manufacturer's protocol (Qiagen) and run on a 1% formaldehyde agarose gel as described[26]. The gel was blotted onto Zeta-Probe GT genomic membrane (Bio-Rad) and hybridized with a radiolabelled hCdc4 probe followed by autoradiography. For Southern blot analysis, 10 μg of DNA was digested with *Sst*I and *Eco*RV, run on an 0.8% agarose gel, blotted and probed with a genomic fragment corresponding to exons 8 and 9.

In vitro binding

Complementary DNAs encoding various hCdc4 isoforms and mutants, β-TrCP and Skp2 were translated *in vitro* into ^{35}S-methionine-labelled proteins by a T7 transcription/translation system (Promega). GST-tagged cyclin E and various cyclin E phosphorylation-site mutants were expressed in baculovirus-infected SF9 insect cells and adsorbed on glutathione beads. Bound proteins were analysed by SDS–PAGE followed by autoradiography. For further details, see Supplementary Information.

In vitro ubiquitination assay

Recombinant SCF complexes containing different Flag-tagged F-box proteins were isolated from transfected 293T cells. Equal amounts of SCF immune complexes were mixed with cyclin E protein for 30 min on ice to allow binding. Aliquots of this mixture were then added to ubiquitination reactions in a total volume of 30 μl containing 15 μg of bovine ubiquitin (Sigma), 0.5 μg of yeast E1 enzyme (Boston Biochem), 1 μg of human 6xHis-Cdc34 purified from bacteria, and an ATP-regenerating system (1 mM ATP, 20 mM creatine phosphate, 0.1 mg ml^{-1} creatine kinase) in ubiquitination reaction buffer[25] supplemented with 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulphonylflouride, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 2 μg ml^{-1} pepstatin. The Cdk2-inhibitor roscovitine (Biomol; 100 μM final concentration) was added to reactions containing dephosphorylated cyclin E as substrate. Reactions were incubated at 30 °C for 2 h, terminated by boiling for 5 min with SDS sample buffer, and analysed by SDS–PAGE followed by immunoblotting using anti-cyclin E antibodies. The ubiquitination assay using p27 as a substrate was performed as described[25].

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Correspondence and requests for materials should be addressed to S.I.R. (e-mail: sreed@scripps.edu). The GenBank accession number for the hCdc4 cDNA encoding the isoform with M_r 110K is AY049984.

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Advances in Brief

hCDC4 Gene Mutations in Endometrial Cancer[1]

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Abstract

Cyclin-dependent kinase 2 activated by cyclin E is involved in the initiation of DNA replication and other S phase functions. Consistent with this role, cyclin E protein accumulates at the G_1-S phase transition and declines during early S phase. This profile of expression is the result of periodic transcription and ubiquitin-mediated proteolysis directed by SCF^{hCdc4}. However, in many types of human tumors cyclin E protein is elevated and deregulated relative to the cell cycle by an unknown mechanism. Here, we show that the F-box protein hCdc4 that targets cyclin E to the SCF (Skp1-Cull-F-box) protein ubiquitin ligase is mutated in at least 16% of human endometrial tumors. Mutations were found either in the substrate-binding domain of the protein or at the amino terminus, suggesting a critical role for the region of hCdc4 upstream of the F-box. *hCDC4* gene mutations were accompanied by loss of heterozygosity and correlated with aggressive disease. The *hCDC4* gene is localized to chromosome region 4q32, which is deleted in over 30% of human tumors. Our results show that the *hCDC4* gene is mutated in primary human tumors and suggest that it may function as a tumor suppressor in the genesis of many human cancers.

Introduction

Cyclin E accumulates at the G_1-S phase transition and is involved in the initiation of DNA replication and other S phase programs (1–4). Its degradation during early S phase is triggered by phosphorylation on residues Thr 62 and/or Thr 380 and is dependent on SCF[3] ubiquitin ligase activity (5–7). SCF ubiquitin ligases are composed of a core complex of Cul1, Rbx1, and Skp1 linked to a variable component known as an F-box protein, which provides substrate specificity. We (6) and others (8, 9) have recently identified hCdc4 (also designated Fbw7 and Ago) as the F-box protein that targets appropriately phosphorylated cyclin E for SCF-mediated ubiquitination. Although cyclin E protein levels are tightly controlled during the cell cycle, in many types of human tumors the level is often elevated and deregulated relative to the cell cycle (10–12), and this phenotype has been associated with poor patient prognosis (13–16). Furthermore, deregulated cyclin E expression can generate tumors in a mouse model (17). Although the basis for cyclin E-mediated tumorigenesis is not known, the fact that constitutive cyclin E expression causes genomic instability (18) suggests a possible mechanism whereby elevated levels of chromosome loss may accelerate LOH. On the other hand, the molecular mechanism(s) by which cyclin E protein becomes deregulated in tumors has remained elusive. We have observed that elevated cyclin E protein levels in tumor-derived cell lines often occurs without a coordinate increase in cyclin E mRNA, suggesting the involvement of a posttranscriptional process (data not shown). We, therefore, first characterized the human *CDC4* gene and then determined whether *hCDC4* gene mutation could be responsible for the cyclin E phenotype observed in human tumors.

Materials and Methods

hCDC4 Genomic Organization. The *hCDC4* genomic locus was identified using the high throughput genomic sequence database. The *hCDC4* gene was found to be contained within BAC clones RP11–555K12 (200147 bp, GenBank accession no. AC023424) and RP11–461L13 (208580 bp, GenBank accession no. AC080078). The internet tool NIX (Nucleotide Identify X[4], United Kingdom HGMP Resource Centre) was used to aid the identification of several untranslated 5′ exons embedded within a predicted CpG island. RT-PCR was used to confirm all exon predictions.

Cell Cycle Analysis. HeLa cells were synchronized by thymidine-nocodazole double block procedure. Treatment with 2 mM thymidine was for 20 h, followed by release for 3 h, then incubation with nocodazole (75 ng/ml) for 12 h. Cells were released from nocodazole, and samples were taken every 1.5 h for 21 h. Cyclin E and PSTAIRE (CDK1 + CDK2) were detected by Western blot using monoclonal antibody HE12 and an anti-PSTAIRE antibody, respectively. hCdc4 protein was detected by immunoprecipitating 500 μg of lysate with an anti-hCdc4 antibody, followed by Western blotting. Cell cycle progression was monitored by fluorescence-activated cell-sorting analysis.

Tumor Analysis. DNA, RNA, and protein were isolated from 51 fresh frozen endometrial adenocarcinomas. Tumors were graded and staged according to International Federation of Gynecologists and Obstetricians guidelines. Protein extracts were prepared in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 μg/ml aprotinin, 1 μg/ml leupeptin-pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM vanadate, and 10 mM NaF]. Phosphorylated cyclin E was detected by subjecting 5–100 μg of lysate (normalized for cyclin E protein levels) to Western blot analysis (7.5% SDS polyacrylamide gels) using the monoclonal antibody HE12. PCR primers used for SSCP analysis are available upon request. All samples displaying aberrant SSCP banding patterns were confirmed by an independent analysis, followed by DNA sequencing. Corresponding formalin-fixed tumor-free tissues were used as controls. LOH was determined for microsatellite markers D4S1554, D4S1572, D4S1586, D4S1607, D4S1615, D4S171, and D4S2915. The PCR primers and conditions used were as described by the manufacturer (Research Genetics). Quantitative RT-PCR for hCdc4 was performed on 100 ng of poly-A+ RNA as a template and primers 5′-ATGGGCCCTGCTCTTCACTTCATGTCC-3′ and 5′-CACTGTGCGTTGTATGCATC-3′ in a 20-cycle PCR reaction (T_{an} = 55°C). Primers specific for human protein phosphatase 1 were used as a control (Stratagene, La Jolla, CA).

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[3] The abbreviations used are: SCF, Skp1-Cull-F-box; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.

[4] Internet address: http:menu.hgmp.mrc.ac.uk/menu-bin/Nix/Nix.pl.

Results

The *hCDC4* Gene Locus. We searched various genome and expressed sequence tag databases to determine the organization of the *hCDC4* gene (see "Materials and Methods"). The *hCDC4* gene locus maps to chromosome region 4q32, which is frequently deleted in a broad spectrum of human tumor types (19) and is composed of 4 untranslated and 13 coding exons spanning approximately 210 kb of the human genome (Fig. 1*a*). RT-PCR was used to confirm exon order using HeLa cell mRNA as a template (data not shown). Search of the expressed sequence tag databases combined with published reports (6, 8, 9, 20, 21) revealed the existence of three primary splice variants (designated α, β, and γ). Ten common 3′ exons are alternatively spliced to three different 5′ coding exons. RT-PCR demonstrated that all three variants are expressed in HeLa cells, although the γ-form was difficult to amplify, suggesting a low abundance (data not shown). Northern blot analysis using probes specific for the various 5′ exons demonstrated that the α-splice variant of *hCDC4* is expressed as a 5.5-kb mRNA, whereas the β- and γ-forms both are expressed as 4-kb mRNAs (data not shown). A CpG island is present 123 kb upstream of the first coding exon of the α-form of hCdc4, and four small noncoding exons are differentially spliced to the α-coding exon (data not shown). The α-form of hCdc4 was also found to be expressed in all human tissues analyzed, whereas the 4-kb mRNA representing the β- and/or γ-forms was present at lower levels, except in skeletal muscle, brain, and to a lesser degree, heart (Fig. 1*b*). Previously, we showed that the α-form is also the predominant *hCDC4* mRNA expressed in tumor-derived cell lines (6), although the β-form could be detected at lower levels. hCdc4 protein levels were found to not vary significantly during the cell cycle in HeLa cells (Fig. 1*c*).

***hCDC4* Gene Analysis in Endometrial Adenocarcinomas.** We next determined whether the *hCDC4* gene is altered in human tumors. Endometrial adenocarcinomas were used in our analysis because previous reports have shown that elevated cyclin E levels occur frequently in these tumors (22) and sufficient tissue was available for protein, RNA, and genomic DNA analysis. Western blot analysis on 51 frozen tumor specimens showed that 8 specimens contained elevated levels of cyclin E protein, and 2 of these tumors showed the phenotype was likely because of genomic amplification of the cyclin E locus (data not shown). Interestingly, we observed an accumulation of phosphorylated cyclin E in seven tumors (Fig. 2*a*). This assessment was based on low mobility on SDS-PAGE and confirmed by phosphatase treatment (Fig. 2*b*). Surprisingly, five of these seven tumors contained only low to moderate levels of cyclin E protein (Table 1).

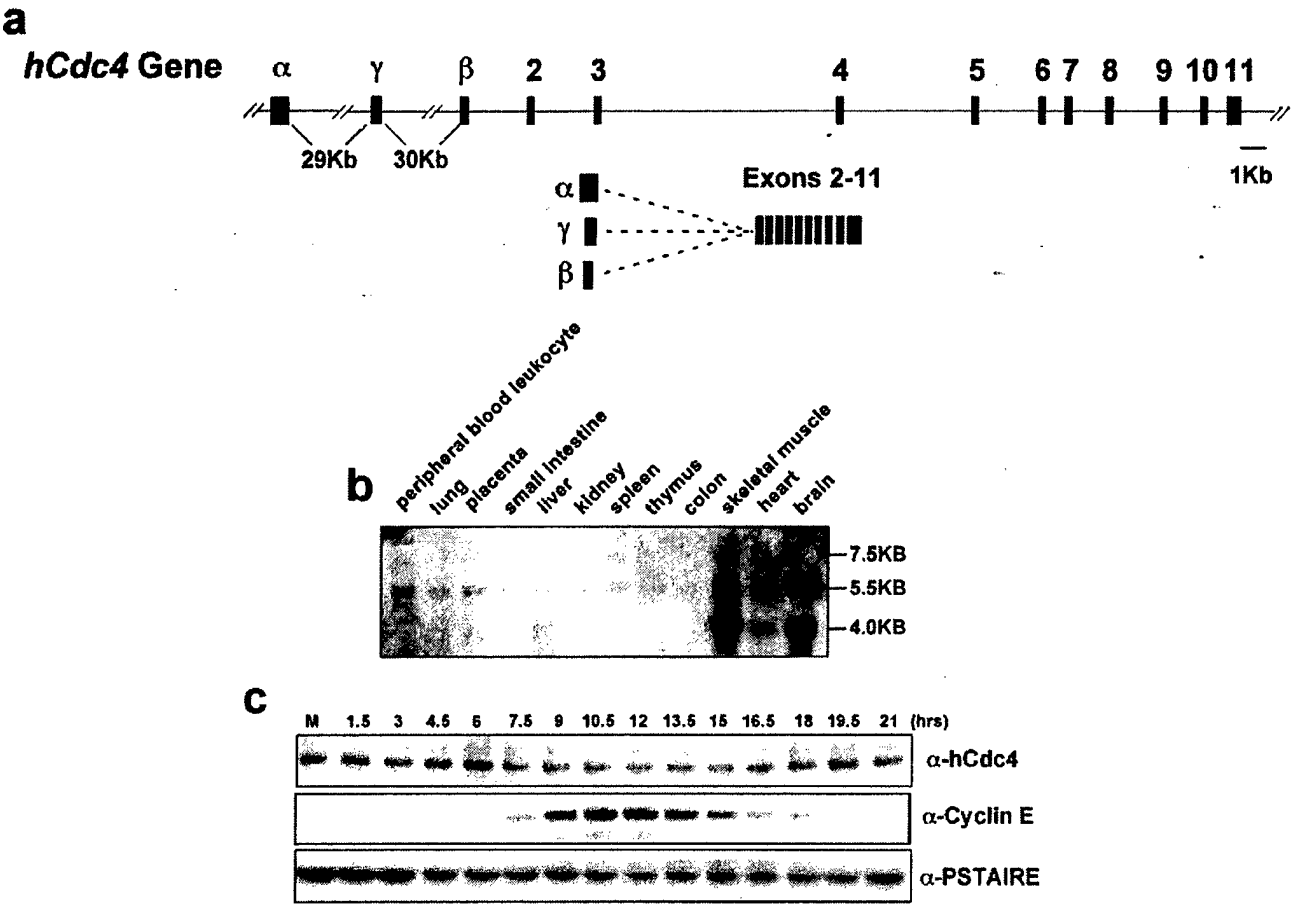

Fig. 1. Genomic organization of the *hCDC4* gene and expression in human tissues and cycling cells. *a*, genomic organization of the *hCDC4* locus localized to chromosome region 4q32. Alternative splicing joins exons 2–11, containing the F-box motif and seven WD40 protein-binding motifs, to one of three different 5′ exons. *b*, tissue blot of hCdc4 in human tissues. A multi-tissue Northern blot was probed with exons 2–11 of the hCdc4 cDNA. The α-form of hCdc4 corresponds to the 5.5-kb band, and the β- and γ-forms correspond to the 4.0-kb band. *c*, *hCdc4* expression during cell cycle progression. HeLa cells were released from M phase arrest, and samples were taken at the indicated time points. hCdc4 protein was detected by immunoprecipitation/Western blotting. Cells enter S phase of the cell cycle at approximately 10.5 h.

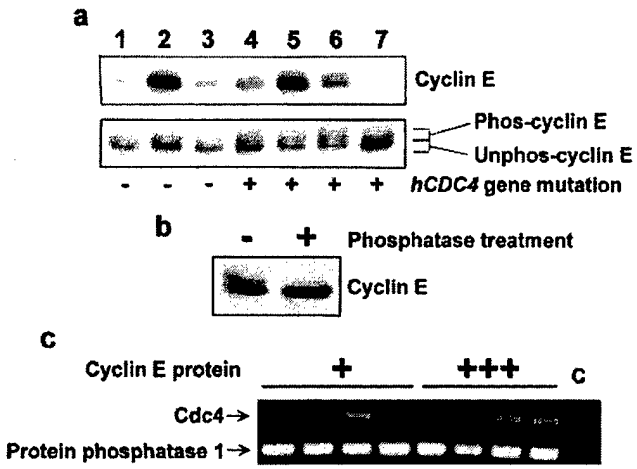

Fig. 2. Analysis of cyclin E and *hCDC4* in endometrial adenocarcinomas. *a*, cyclin E protein levels and phosphorylation status in endometrial adenocarcinomas. Cyclin E protein levels in tumors were determined by Western blotting with anticyclin E antibody (*top*). Cyclin E levels were normalized, and lysates were run on 7.5% SDS-PAGE gels and probed with anti-cyclin E antibody (*bottom*). A slower migrating band is observed in tumors that contained inactivating mutations of hCdc4 (*Lanes 4–7*). *b*, phosphatase treatment of lysate displaying altered mobility of cyclin E. Lysate from tumor in *Lane 6* of *a* was treated with λ-phosphatase for 60 min on ice, run on a 7.5% SDS-PAGE gel, and subjected to Western blot analysis with anti-cyclin E antibody. *c*, RT-PCR analysis of *hCdc4* expression in endometrial adenocarcinomas. RT-PCR reactions were performed on poly-A+ RNA isolated from tumors to determine level of hCdc4 expression as compared with control protein phosphatase 1.

Northern blotting and RT-PCR analysis revealed that *hCDC4* mRNA was expressed over a range of 2–4-fold in all tumors analyzed, and the level of expression did not correlate with cyclin E protein level or phosphorylation status (Fig. 2*c* and data not shown). We performed SSCP analysis of the *hCDC4* gene from tumors that exhibited either elevated cyclin E levels or phosphorylated cyclin E or both (13 of 51). In addition, *hCDC4* SSCP analysis was performed on 15 control tumors that had neither elevated cyclin E levels nor phosphorylated cyclin E. Aberrant SSCP banding patterns, indicative of mutations, were observed in eight tumors (Fig. 3 and Tables 1–3). Interestingly, six of eight tumors with *hCDC4* gene mutations also accumulated phosphorylated cyclin E, and only two of these tumors contained elevated cyclin E protein levels (Table 1 and Fig. 2). A wild-type SSCP banding pattern was observed for 14 of 15 control tumors (with neither elevated nor phosphorylated cyclin E). DNA sequencing demonstrated that six of eight mutations occurred within the seven WD40 repeat domains of hCdc4 that are proposed to be involved in substrate recognition (Table 3). Four mutations introduced a stop codon within the WD40 repeat region. Previously, we have shown that truncations within the hCdc4 protein that delete WD40 repeats eliminate cyclin E binding (6). Furthermore, two missense mutations occurred at Arg residues that are conserved in the Cdc4 homologues of *Saccharomyces cerevisiae*, *Drosophila*, *Caenorhabditis elegans*, and human (6, 9). On the basis of the putative structure of the Cdc4 protein, one of these Arg residues (codon 465) is located on the surface of the β-propeller structure that is proposed to be involved in phosphorylation-dependent recognition of cyclin E (9). Mutation of this residue has previously been shown to abolish cyclin E binding *in vitro* (9). One mutation (Glu→Tyr, codon 124) occurred outside the WD40 repeat regions, in the 5′ exon of the α-form of h*CDC4*. Another mutation was localized to the 5′ exon of the β-form (GTT→ATT, codon 23). This mutation was obtained from one of the "control" tumors that had neither elevated nor phosphorylated cyclin E. Interestingly, of the eight *hCDC4* gene mutations detected, the six that were localized to the WD40 repeat region and, therefore, presumably prevented substrate binding occurred in tumors with an accumulation of phosphorylated cyclin E. In contrast, the two tumors that contained *hCDC4* gene mutations that were localized to the aminoterminal region of hCdc4 and predicted not to affect substrate binding did not accumulate phosphorylated cyclin E (Tables 1 and 3). Thus, the accumulation of phosphorylated cyclin E may depend on the inability of hCdc4 to bind substrate. In addition, because not all of the tumors were subjected to SSCP analysis (only those showing elevated cyclin E levels or increased cyclin E phosphorylation and 15 controls), it is conceivable that additional *hCDC4* mutations were missed. Thus, eight *hCDC4* mutations in 51 tumors is likely a minimal estimate. No mutations were detected in paired normal tissue DNA corresponding to any tumor, confirming that all of the *hCDC4* gene mutations in tumors were of somatic origin (Fig. 3).

The SSCP data suggested that most of the tumors that contained *hCDC4* gene mutations did not retain a wild-type allele of *hCDC4*, as indicated by the absence of a wild-type banding pattern (Fig. 3 and data not shown). We examined these tumors further for LOH of several markers surrounding the *hCDC4* gene on chromosome region 4q32. Evidence of LOH was observed in seven cases where informative heterozygosities were apparent in matched nontumor samples, confirming the loss of the remaining *hCDC4* allele (Fig. 3). A single tumor was noninformative at all loci analyzed.

Discussion

Our results are the first to demonstrate *hCDC4* gene mutations in primary human tumors and suggest that the F-box protein hCdc4 may function as a tumor suppressor in the genesis of endometrial carcinoma. In most cases, *hCDC4* gene alterations were accompanied by a coordinate LOH of the remaining wild-type allele consistent with Knudson's (23) "two-hit" hypothesis of tumor suppressor genes. *hCDC4* gene mutations were significantly correlated with high-grade (G3) tumors ($P = 0.05$, χ^2 test) and trended toward high-stage tumors ($P = 0.104$). Furthermore, within a subset of patients ($n = 19$) analyzed for evidence of pelvine lymph node involvement, there was a significant correlation with hCDC4 mutations [*i.e.*, 100% (3 of 3) with tumors having *hCDC4* mutations had positive lymph nodes compared with 25% (4 of 16) without mutations ($P = 0.036$, Fisher's exact test)]. Although the data are limited, they suggest that hCdc4 gene mutations may correlate with particularly aggressive disease.

hCDC4 may also be involved in the genesis of many other tumor types because deletion of chromosome region 4q32 has been

Table 1 *Analysis of endometrial adenocarcinomas*

No.	Grade/stage	↑ Cyclin E	Phos-cyclin E	*hCDC4* mutation	Lymph node[a]
1	G2/1.3	+	–	–	n/a
2	G3/3.1	+	–	–	–
3	G3/1.3	+	–	–	–
4	G3/4.2	+	–	–	n/a
5	G3/2.2	+	–	–	n/a
6	G3/3.3	+	–	+	+
7	G3/3.3	+	+	+	+
8	G3/1.3	+	+	+	n/a
9	G2/3.3	–	+	+	+
10	G3/1.3	–	+	+	n/a
11	G3/1.3	–	+	+	n/a
12	G3/n/a	–	+	+	n/a
13	G3/2.1	–	+	–	–
14–51	Average grade, 1.4 Average stage, 2.4	–	–	1/15	4/13

[a] Positive pelvine lymph nodes. n/a, not available.

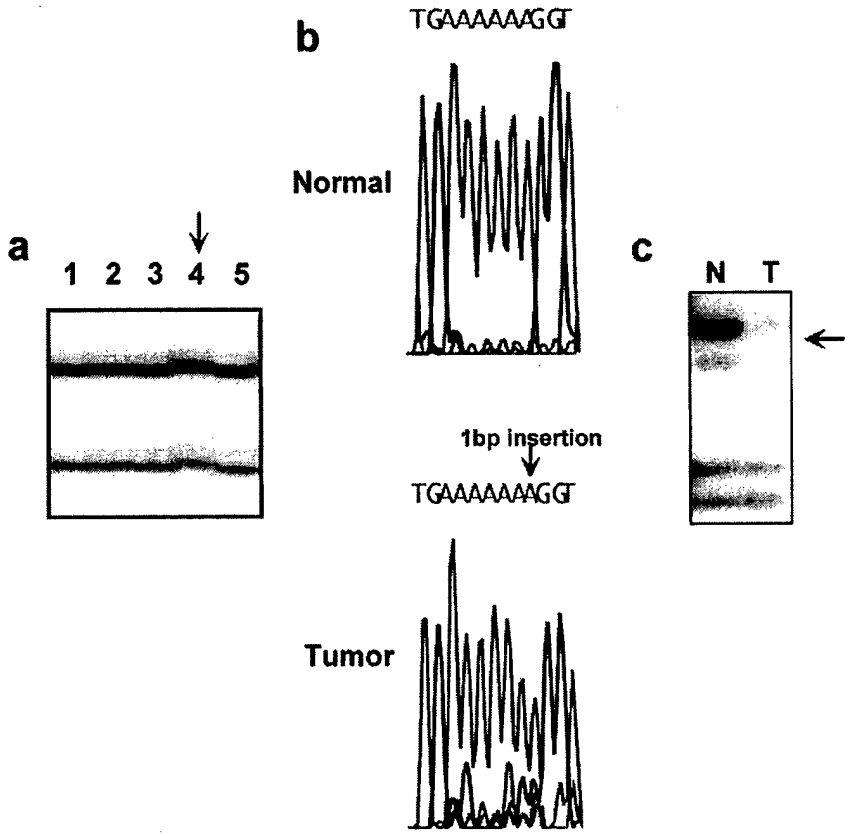

Fig. 3. Genetic analysis of *hCDC4* gene in endometrial adenocarcinoma. *a*, SSCP gel. An altered banding pattern and absence of a normal banding pattern is observed in tumor from *Lane 4* (corresponding to tumor 6 in Table 1). *b*, sequencing chromatogram of tumor displaying altered SSCP banding pattern. A single base (A) insertion at codon 472 in exon 8 introduces a stop codon at codon 476, resulting in a truncated protein. *c*, LOH analysis. PCR-based analysis of microsatellite locus D4S1586 near chromosome region 4q32 demonstrates loss of upper allele and retention of a single mutant allele of *hCDC4*.

reported in 31% of all neoplasms, including 67% of lung cancers, 63% of head and neck cancers, 41% of testicular cancers, and 27% of breast cancers (19). Remarkably, the 16% frequency of *hCDC4* gene alterations detected in this study is comparable with the frequency (17%) of 4q32 deletion reported for endometrial adenocarcinomas (19). Further study is necessary to determine whether the frequency of 4q32 LOH in these other tumor types corresponds to the involvement of *hCDC4* or a yet to be identified tumor suppressor gene.

It is currently unclear how alteration of hCdc4 function contributes to tumorigenesis. Tumors containing *hCDC4* gene mutations within the WD40 binding domains accumulated phosphorylated cyclin E, but four of six of these tumors expressed a low to moderate level of cyclin E protein. These results are unexpected, in that impairment of cyclin E degradation processes would be expected to result in an increase in the steady-state level of cyclin E protein in its phosphorylated form. This was confirmed by experiments where an F-box-deleted and, thus, presumably dominant negative form of hCdc4 was introduced into wild-type cells by adenoviral transduction (6). We have found that cyclin E mRNA levels are not down-regulated in tumors containing *hCDC4* gene mutations (data not shown), eliminating a potential role for transcriptional regulation. Alternatively, other proteolytic mechanisms of cyclin E, possibly the Cul3 pathway (24), could be up-regulated in tumors with *hCDC4* gene mutations, but this hypothesis awaits further investigation.

Normally, cyclin E accumulation is limited to a narrow window at the G_1-S phase boundary of the cell cycle (25). Deregulated cyclin E-associated kinase activity relative to the cell cycle and not elevated cyclin E protein *per se* may be the critical link between *hCDC4*

Table 2 *Summary of analysis*

	Tumor grade (G3/G2)	*hCDC4* gene mutation
↑ Cyclin E protein (8)	7/1	3/8[a]
Phos-cyclin E (7)	6/1	6/7
Neither (38)	16/22	1/15

[a] Two of these tumors also contained phos-cyclin E.

Table 3 *hCDC4 gene mutations in endometrial carcinomas*

No.	Mutation	Codon[a]	Result	LOH 4q32
6	GAG→TAT	124	Glu→Tyr	+
7	AAA→(TC)A (2-bp insertion)	371	Lys→Ser, term codon 376	+
8	CGA→TGA	367	Arg→Ter	+
9	CGT→CAT	465	Arg→His	ni[b]
10	CGA→CAA	479	Arg→Gln	+
11	CGA→TGA	658	Arg→Ter	+
12	AGA→A(A)G (1-bp insertion)	472	Arg→Lys, term codon 476	+
14	GTT→ATT	23[c]	Val→Ile	+

[a] Codon number based on α-form of *hCDC4*.
[b] ni, noninformative at all loci analyzed.
[c] Mutation in 5'-exon of β-form of *hCDC4*.

mutation and tumorigenesis. In support of this hypothesis, we have found that cyclin E protein is deregulated in a breast tumor-derived cell line containing a *hCDC4* gene mutation.[5] Additional studies are currently underway to determine whether cyclin E expression is deregulated in tumors containing inactivating mutations of *hCDC4*.

One surprising finding of this study is that mutations in the unique domains of either of two alternatively spliced variants of *hCDC4* are associated with tumorigenesis. α-Variant-specific mutation is consistent with the observation that this form is predominant in tumors and tumor-derived cell lines, but β-variant-specific mutation is difficult to rationalize in this context. These data, however, suggest that the functions of these two alternatively spliced variants are not redundant.

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